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Autographa californica M Nucleopolyhedrovirus ProV-CATH is Activated during Infected Cell Death

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V-CATH, a cathepsin L-like cysteine protease encoded by the baculovirus *Autographa californica* M nucleopolyhedrovirus, has been shown to play an essential role in host liquefaction. Similar to cellular cathepsin L, V-CATH is synthesized as an inactive proenzyme and is activated by cleavage of the propeptide. Previous studies indicated that removal of the propeptide was rapid, occurring as soon as the protein could be detected by Western blot, 22 h postinfection. We found, however, that these results reflected artifactual processing of the proenzyme. When the protease inhibitor E-64 was used to prevent this aberration, we found that proV-CATH accumulated in infected cells and activation did not begin until the onset of cell death, at approximately 80 h postinfection. Western blot analysis of fractions of live and dead cells isolated by fluorescence-activated cell sorting revealed that mature V-CATH was found only in dead cells. The regulation of activation of proV-CATH, therefore, was quite different from that of cellular cathepsins. Acridine orange staining revealed that lysosome integrity was lost in dead cells, an occurrence that could lead to the activation of proV-CATH by lysosomal proteases.

Key Words: AcMNPV V-CATH activation; baculovirus cathepsin.

INTRODUCTION

Autographa californica M nucleopolyhedrovirus (AcMNPV) is the type species of the *Nucleopolyhedrovirus* genus of the family *Baculoviridae* (Blissard *et al.,* 2000). It is an enveloped, double-stranded DNA virus that infects a select range of lepidopteran species. Infection of the insect host normally begins when a larva ingests plant material contaminated with virus in the form of occlusion bodies, or polyhedra—proteinaceous bodies 0.15–15 μm in diameter that protect the enclosed virions from environmental damage (Volkman, 1997). The polyhedra dissolve in the alkaline juices of the insect midgut lumen, releasing the virions. These occlusion-derived virions (ODV) then enter the columnar cells of the midgut epithelium where they initiate infection (Volkman, 1997). Once infection has been established in the midgut, it is spread to the other tissues by a second viral morphotype, referred to as budded virus (BV) (Monsma *et al.,* 1996). The newly infected cells, in turn, begin to produce additional BV and then, in the late and very late phases of infection, make ODV and polyhedra.

The dispersal of polyhedra and, thus, the horizontal transmission of AcMNPV infection, is facilitated by the liquefaction (sometimes called "melting") of the host cadaver following death (Volkman and Keddie, 1990). In a dramatic transformation, the insect corpse is reduced to a viscous mass of polyhedra-laden fluid. Notably, this process occurs as a discrete event at the end of infection, not as a gradual process throughout infection. Host liquefaction depends on the presence of two viral gene products: chiA, a chitinase (Hawtin *et al.,* 1997), and V-CATH, a cysteine protease. The two genes are present in members of the *Nucleopolyhedrovirus* as well as the *Granulovirus* genera of baculoviruses, and they are monophyletic, suggesting that they were acquired prior to the divergence of the two genera (Kang *et al.,* 1998). This hypothesis is supported by the recent finding that V-CATH is not processed properly in the absence of chiA, an observation that provides an explanation for the absence of liquefication of host tissues infected with chiA-deficient mutants (Hom and Volkman, 2000).

V-CATH resembles the lysosomal protease, cathepsin L, in sequence (Slack *et al.,* 1995). Bromme and Okamoto (1995) characterized the enzymatic activity of V-CATH and found that the pH optimum was between 5.0 and 5.5, but that the enzyme demonstrated measurable activity at pH 7.0. Moreover, they found the substrate specificity was closer to that of cathepsin B than cathepsin L even though the sequence of V-CATH more closely resembled that of cathepsin L. In addition, they found that the substrate binding pocket of V-CATH was less restricted sterically than those of cathepsins L, S, or B.

Similar to mammalian cathepsins and other members

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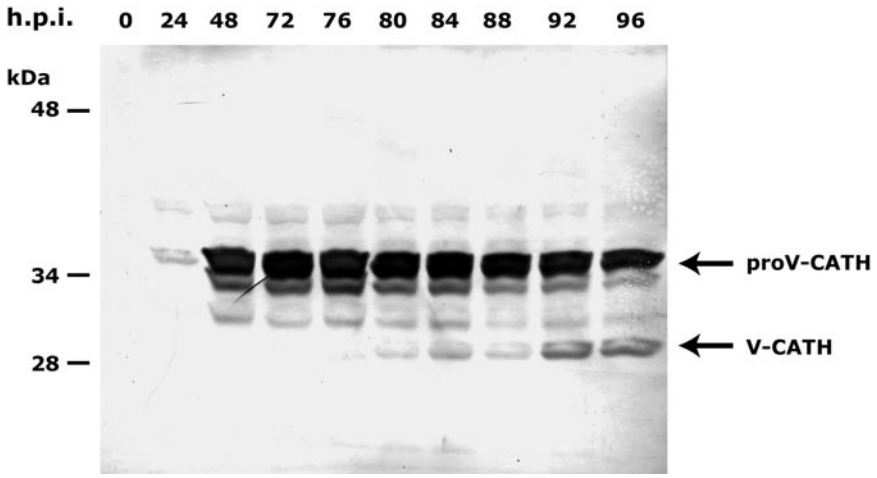

FIG. 1. proV-CATH processing in wild-type AcMNPV-infected cells. Cell lysates were prepared from Sf9 cells infected with wild-type AcMNPV and analyzed by Western blot. E-64 was included in cell lysis buffer to prevent induction of processing of proV-CATH by SDS. Numbers on top indicate hours postinfection (h.p.i.). Molecular weights of protein standards are indicated on the left. V-CATH and proV-CATH are identified by arrows, as labeled, on the right.

of the papain superfamily of proteases, V-CATH is synthesized as an inactive proenzyme, designated proV-CATH (approximately 36 kDa in size), and is activated by proteolytic removal of the inhibitory propeptide yielding the mature protease (approximately 28 kDa). Mammalian cathepsin zymogens are targeted to lysosomes where proteolytic cleavage and activation take place; hence, processing occurs within hours of synthesis of the zymogens (Kirschke *et al.,* 1998). It was previously reported that removal of the proV-CATH propeptide is a rapid process *in vivo* (Slack *et al.,* 1995), similar to that of mammalian cathepsins, and that mature V-CATH is present in infected cell lysates as soon as the enzyme can be detected by Western blot [22 h postinfection (h.p.i.)]. We have found, however, that proV-CATH processing can be triggered by the chaotropic agent, sodium dodecyl sulfate (SDS) (Hom and Volkman, 1998), a common reagent used in analytical methods such as polyacrylamide gel electrophoresis (PAGE) and Western blot preparation. This artifactual processing can result in a variety of experimental complications, but it can be prevented by the addition of a cysteine protease inhibitor, such as E-64, to the buffers used during sample preparation.

Because the previous studies of V-CATH did not include this preventive measure, we sought to determine whether SDS-induced processing affected the results of those experiments. We discovered that this was indeed the case: proV-CATH accumulated within infected cells, not mature V-CATH. Hence, the regulation of activation of proV-CATH was different from that of cellular cathepsins. In addition, we found that the timing of activation correlated with cell death, suggesting that physiological changes incurred by infected cells during the onset of cell death triggered the activation of proV-CATH.

RESULTS

Accumulation of V-CATH in infected cells

V-cath is classified as a late AcMNPV gene. In previous studies of proV-CATH expression and processing (as assessed by Western blots), V-CATH was first detected in lysates of infected cells at 22 h.p.i. It was reported that nearly all of the enzyme was present in the mature (processed) form and that there was very little proV-CATH present (Slack *et al.,* 1995). Because those studies did not incorporate protease inhibitors to prevent the processing of proV-CATH induced by SDS, however, it was possible that those results did not accurately reflect the state of the enzyme in the infected cells. We reexamined, therefore, the temporal regulation of proV-CATH with this precaution in place. We found no mature V-CATH in infected cells until approximately 80 h.p.i. (Fig. 1); thus, V-CATH appeared to accumulate in infected cells as the inactive proenzyme and not as the mature, active enzyme.

Activation of proV-CATH

The finding that maturation of proV-CATH occurred 3–4 days postinfection (d.p.i.) was intriguing because this coincided with the onset of death among infected cells in culture. To confirm that processing of the proenzyme to the 28-kDa mature form was indicative of activation and not nonspecific degradation that might occur in dead and dying cells, we conducted enzyme activity assays in conjunction with Western blots in time-course experiments. The enzyme activity assays were conducted under neutral conditions to prevent activation of proV-CATH by acidic conditions (Bromme and Okamoto, 1995) and also to minimize the background activity of lysosomal proteases, most of which are rapidly inactivated at pH

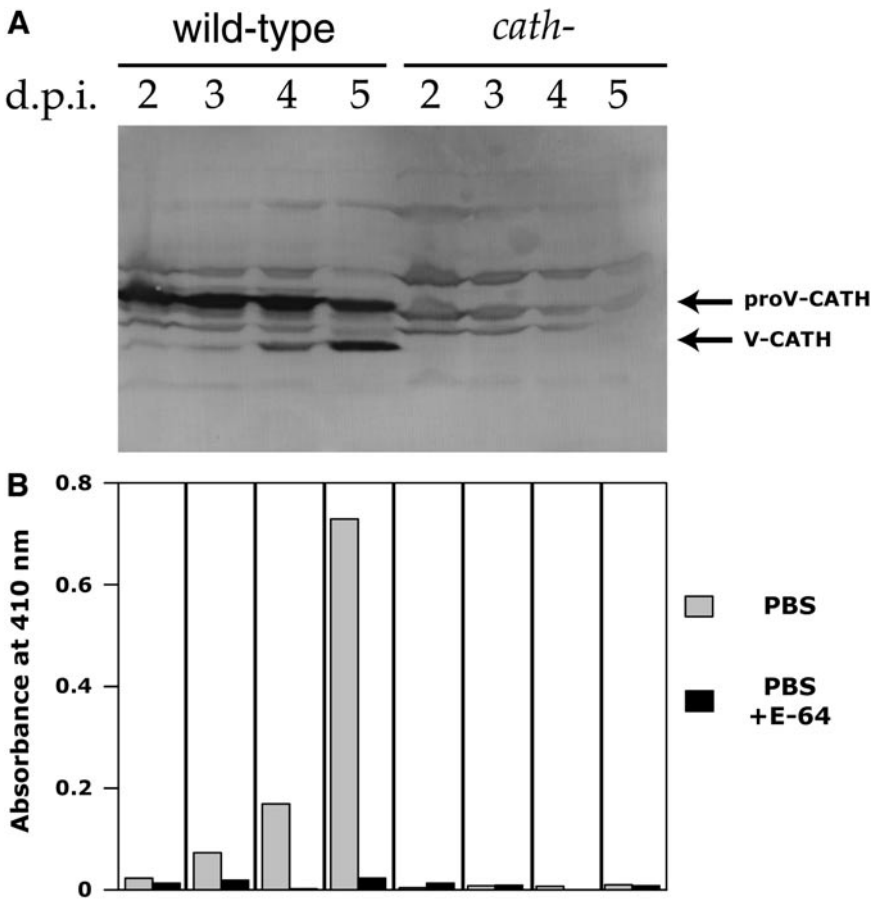

FIG. 2. Correlation of proV-CATH processing and V-CATH activation in time-course experiments. Paired samples of Sf9 cells infected with wild-type or *cath*-AcMNPV and were taken at the indicated day postinfection (d.p.i.) and analyzed (A) by Western blot following addition of sample buffer containing E-64 and (B) in protease assays, with and without addition of E64. V-CATH and proV-CATH are identified by arrows, as labeled, on the right in (A).

7.0. V-CATH, in contrast to most lysosomal proteases, is active at pH 7.0, although suboptimally (Bromme and Okamoto, 1995). The results of such assays were that there was little proteolytic activity prior to day 3 in wild-type infected cells, but on days 3, 4, and 5 there was a progressive increase in activity that coincided with the appearance of the 28-kDa protease (Figs. 2A and 2B). This activity was sensitive to E-64, and no activity occurred when cells were infected with *cath*$^-$ (C$^-$DZ1) virus. These findings confirmed that proV-CATH was being activated rather than degraded.

The finding that proV-CATH was activated so late in infection implied that activation and cell death could be related processes. To investigate this possible connection, we used flow cytometry to isolate live and dead cell fractions from an infected culture, based on the inability of dead cells to exclude the vital stain, propidium iodide. In Western blots of the live and dead cell fractions (Fig. 3), mature V-CATH was found only in the dead cell fraction, and the live cell fraction was found to contain only proV-CATH. This confirmed that proV-CATH activation was closely tied to cell death.

This discovery raised the question of whether proV-CATH activation might actually contribute to cell death. We compared, therefore, the rates of cell death in cultures infected with either wild-type or *cath*$^-$ virus. If proV-CATH activation contributed to cell death, one would expect to see wild-type infected cells dying sooner than those infected with the *cath*$^-$ virus, and this is what we saw (Fig. 4). Both Sf9 and TN368 cells infected with wild-type virus, whether they were grown in serum-free media (not shown) or in media with serum, died slightly sooner than cells infected with *cath*$^-$ virus. Thus, proV-CATH expression, and presumably activation, could induce premature cell death after several days of infection.

Correlation of proV-CATH activation with lysosomal leakage

AcMNPV-infected cells generally undergo necrotic cell death as virus-encoded gene products prevent apoptosis (Clem *et al.,* 1991; Manji *et al.,* 1997). One common feature of necrotic cell death is the loss of intracellular

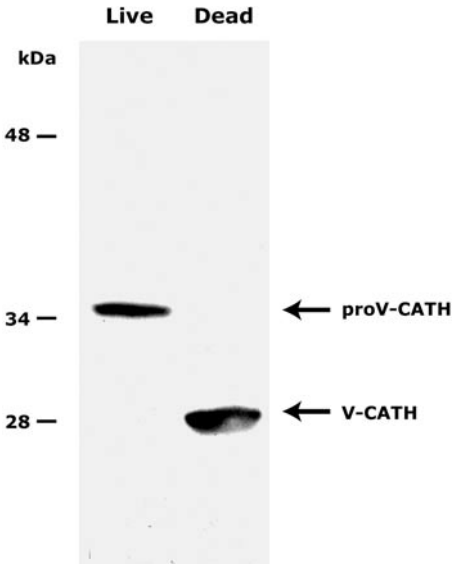

FIG. 3. Mature V-CATH is associated with dead cells. Sf9 cells infected with wild-type AcMNPV were stained with propidium iodide to identify those cells which had died. Fluorescence-activated cell sorting was used to isolate live and dead cell fractions, and these fractions were analyzed by Western blot, shown above. Numbers on the left indicate the molecular weights of protein standards.

compartmentalization—various organelles undergo osmolytic destruction and release their contents into the cytoplasm (Cohen, 1994). Because lysosomes contain proteases that could, in principle, activate proV-CATH, we sought to evaluate the integrity of lysosomes in these dying cells. We stained infected cells at 92 h.p.i. simultaneously with the vital stain trypan blue and the dye acridine orange, which localizes to lysosomes in healthy cells (Brunk *et al.,* 1995). In cells that were alive, bright

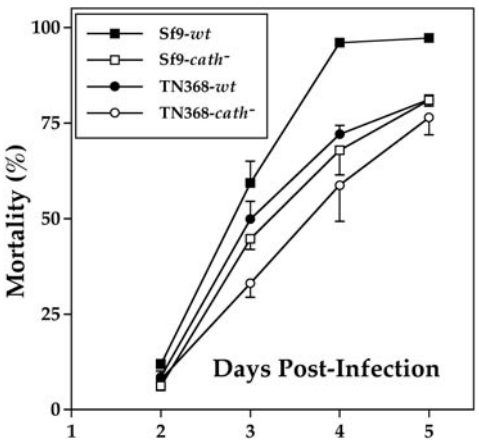

FIG. 4. Comparative cell mortality in *cath*$^-$ and wild-type virus-infected cultures. Sf9 and TN368 cells were infected with either wild-type (*wt*) or *cath*$^-$ virus. At the designated times postinfection, the cells were counted in triplicate and the percentage of dead cells noted. The means and standard deviations (positive for *wt* and negative for *cath*$^-$) are indicated. The experiments were repeated twice with similar results.

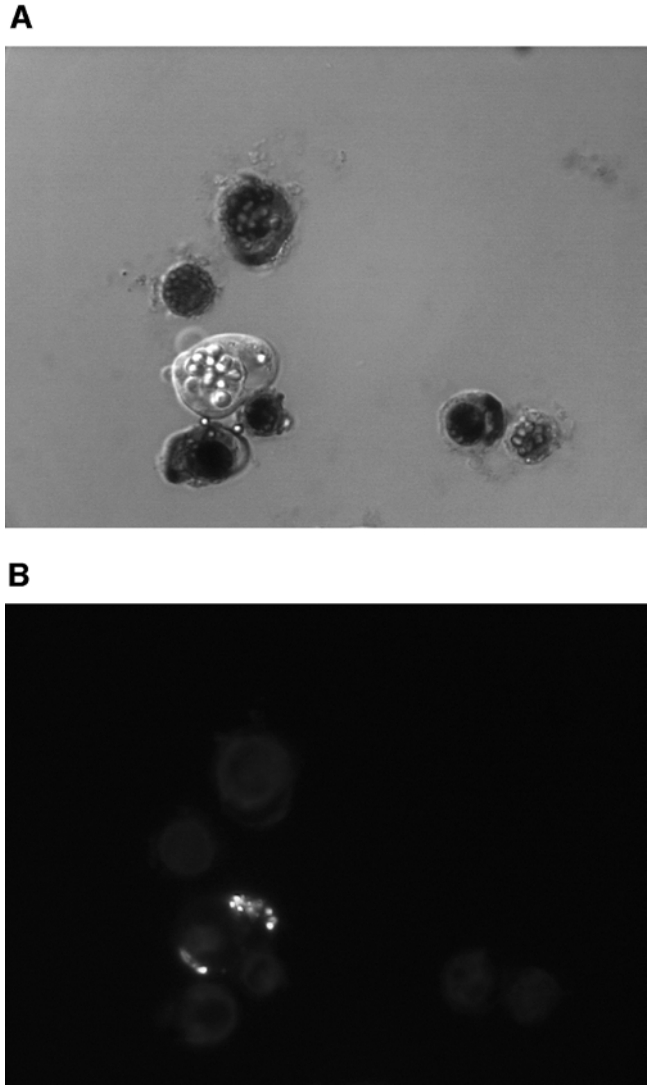

FIG. 5. Lysosomal staining of AcMNPV-infected cells. Sf9 cells were allowed to adhere to glass coverslips and were infected with wild-type AcMNPV. At 92 h.p.i., the cells were stained with acridine orange and trypan blue. Dead cells stain positive for trypan blue and appear as dark bodies in phase contrast microscopy (a). Acridine orange staining of lysosomes appears as bright cytoplasmic foci when viewed through the fluorescein channel (b).

staining of the lysosomes was observed (Fig. 5). In contrast, little or no lysosomal staining occurred in cells that had died, indicating that the membranes of these organelles were no longer intact.

Release of V-CATH from infected cells

Initial attempts to characterize V-CATH included measurements of protease activity in the conditioned medium of infected and uninfected Sf9 cells as late as 4 d.p.i. (Rawlings *et al.,* 1992); no increase in activity was observed in infected cultures. To determine whether this lack of activity was due to a lack of protease secretion or to the secretion of inactive proenzyme, we collected

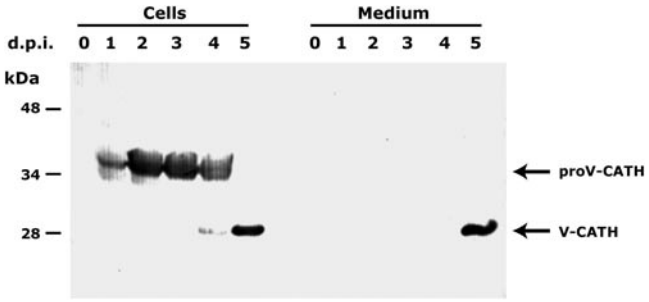

FIG. 6. V-CATH is released from cells following activation. Sf9 cells were infected with wild-type AcMNPV and grown in Excell 401 serum-free medium. Samples of cells (left lanes) and medium (right lanes) were harvested 0–5 d.p.i. and analyzed by Western blot. Numbers on the left indicate the molecular weights of protein standards. Arrows on the right identify V-CATH and proV-CATH, as labeled.

infected cell medium 0–5 d.p.i. and analyzed the samples by Western blot (Fig. 6). No V-CATH was detected in the medium until 4 d.p.i., and only the mature form was detected. Thus, release of V-CATH into the surroundings occurred after cell death.

DISCUSSION

In the original studies of proV-CATH regulation, it was reported that the protease was present in the mature form as soon as the protein could be detected by Western blot. These findings were supported by *in vitro* assays which showed high levels of proteolytic activity in infected cell lysates (Slack *et al.,* 1995) and were consistent with the kinetics of mammalian cathepsin synthesis and activation (Kirschke *et al.,* 1998). Our finding that SDS triggers proV-CATH processing, however, led us to reexamine these findings (Hom and Volkman, 1998). We have found that proV-CATH accumulates in infected cells as the inactive proenzyme, not as the mature enzyme. Hence, the regulation of proV-CATH activation is different from that of its cellular homolog, cathepsin L. Activation was not observed until 3 d.p.i., 2 days later than originally reported. The *in vitro* activity observed previously was likely the result of V-CATH activation that occurred during the course of the assay, owing to the prolonged incubation of lysates in acidic buffer (pH 5.4) containing 3 M urea, which (similar to SDS) has strong chaotropic properties (Slack *et al.,* 1995).

Western blot analysis of live and dead cell populations revealed that mature V-CATH was associated only with dead cells. Two distinct pathways of cell death are currently recognized: apoptotic and necrotic. AcMNPV-infected cells generally undergo necrotic cell death as virus-encoded gene products prevent apoptosis (Clem *et al.,* 1991; Manji *et al.,* 1997). In AcMNPV-infected cells, host macromolecular synthesis is shut down 12–15 h.p.i. (Ooi and Miller, 1988), and cells begin to die days later. Although the specific reasons for AcMNPV-infected cell death are not known, in general, acute disruption of cellular metabolism (such as cessation of macromolecular synthesis) leads to necrotic cell death. Physiological changes associated with necrotic cell death include ATP depletion and reduction in pH, ion dysregulation including an increase in Ca^{2+} concentration, mitochondrial and cellular swelling, loss of intracellular compartmentalization, activation of degradative enzymes, plasma membrane failure, and cytolysis (Lemasters *et al.,* 1998). Our results suggest that some aspect of these changes lead to the activation of proV-CATH and that infected cells containing proV-CATH die sooner than those that do not. One possible mechanism for proV-CATH activation was suggested by the observed loss of lysosomal integrity during the course of cell death, as determined by acridine orange staining, releasing proteases that possibly could initiate cleavage and activation of proV-CATH (Vernet *et al.,* 1991).

The lysosomal cathepsins are powerful enzymes; regulation of the proteolytic activity of these enzymes is necessary to prevent damage to the cell's biosynthetic machinery, and the activation of cathepsins only within acidic vesicles is one way to protect cellular processes. Moreover, most cathepsins are unstable and only weakly active (if at all) at neutral pH; thus, they are optimized to function within acidic intracellular vesicles. In addition, these enzymes are most active in a reducing environment, and endosomes accumulate cysteine to maintain such an environment. Finally, cytoplasm and extracellular spaces are endowed with natural inhibitors of these enzymes (Chapman *et al.,* 1997). These regulatory features may explain why mammalian corpses are not quickly degraded by cathepsins escaping from disrupted lysosomes following death. The fact that V-CATH is active under these conditions suggests that it is regulated differently. Herein, we have provided experimental evidence that proV-CATH's activation is also regulated differently. In retrospect, given that the function of V-CATH is to degrade infected caterpillar cadavers, it is not surprising that its binding pocket is sterically less restricted than those of mammalian cathepsins (Bromme and Okamoto, 1995) nor that its activation is triggered by the death of its host. Necrotic cell death is complicated, however, and many physiological changes occur. Which of them specifically activates proV-CATH, directly or indirectly, remains to be determined.

MATERIALS AND METHODS

Cells and virus

Spodoptera frugiperda (Sf9) cells were grown in suspension at 28°C in Vaughn's modified Grace's medium (JRH Biosciences) supplemented with 10% fetal bovine serum (Gemini BioProducts) and 0.1% Pluronic F-68, or in Excell 401 or 420 serum-free medium (JRH Biosciences) supplemented with 0.1% Pluronic F-68. For some experiments, cells were grown in Excell 420 supplemented with 10% fetal bovine serum and 0.1% Pluronic F-68.

Trichoplusia ni (TN368) cells were grown in suspension at 28°C in TNMFH supplemented with 10% fetal bovine serum and 0.1% Pluronic F-68.

A *v-cath*-deficient virus (C^-DZ1) was produced in the following manner. The *Xba*I-*Bam*HI fragment (filled in with Klenow) of pAcDZ1 containing the *Escherichia coli lac*Z gene under the control of the *Drosophila hsp*70 promoter was inserted into the *Bst*BI site (also filled in with Klenow) of the *v-cath* gene in pSK^+*Bam*HI-E, a plasmid containing the complete *chiA* and *v-cath* genes (Hom and Volkman, 2000), to create pSK^+c^-DZ1. To produce C^-DZ1, pSK^+c^-DZ1 and wild-type AcMNPV DNA (strain L1) were cotransfected into Sf9 cells. C^-DZ1 virus was purified by selecting for blue plaques over two courses of plaque purification followed by two rounds of endpoint dilution. To confirm that the expected changes were present in the recombinant virus and that the *chiA* gene was still intact, a fragment of *Bam*HI-digested C^-DZ1 genomic DNA containing the region of interest was ligated into pBluescript KS and subjected to automated DNA sequencing.

AcMNPV wild-type (strains E2 and L1) (Smith and Summers, 1978; Lee and Miller, 1978) and C^-DZ1 budded viruses were propagated in adherent Sf9 cultures and titered by immunoplaque assay (Volkman and Goldsmith, 1982). All reagents were purchased from Sigma Chemical Co., except as noted.

Time course of proV-CATH processing

Sf9 cells were allowed to settle in tissue culture dishes for 1 h prior to infection with wild-type E2 virus [multiplicity of infection (m.o.i.) = 10]. Cells were incubated with viral inoculum on a rocking platform for 1 h. The inoculum was then removed and replaced with fresh medium. At the appropriate time postinfection, the cells were resuspended by gentle pipetting. The cells were pelleted by centrifugation for 10 min at 1000 *g* and resuspended in cell lysis buffer [25 mM Tris, pH 7.5, 100 mM NaCl, 0.5% (v/v) Nonidet P-40 (NP-40), 30 μg E-64 (Boehringer Mannheim) per millliliter].

One-third volume of 4× SDS–PAGE sample buffer [200 mM Tris, pH 6.8, 8% (w/v) SDS, 400 mM dithiothreitol, 20% (v/v) glycerol, 0.1% bromphenol blue, 30 μg E-64 per milliliter] was added to each sample. After boiling for 5 min, the samples were loaded onto a 12% polyacrylamide gel for electrophoresis. Following separation, the proteins were transferred to Immobilon P membrane (Millipore) for Western blot analysis.

The blots were incubated for 1 h in blocking buffer [Tris-buffered saline (TBS), 0.5% NP-40, 0.1% Tween 20, 0.5% bovine serum albumin, 2.3% nonfat dried milk, 0.01% thimerosal], followed by a 1.5-h incubation in rabbit V-CATH antiserum (Slack *et al.,* 1995) diluted in blocking buffer (1.5:2000). After a 15-min rinse in TBS, the blots were incubated for 1 h in horseradish peroxidase conjugated anti-rabbit antibody (Bio-Rad) diluted 1:3000 in blocking buffer. Antibody binding was detected using the SuperSignal chemiluminescent system (Pierce Chemical Co.) or TMB membrane peroxidase substrate (Kirkegaard & Perry Laboratories).

Time course of V-CATH activation

In separate flasks, 4×10^7 log phase Sf9 cells in 23 ml serum-free medium were infected with either wild-type L1 or C^-DZ1 at an m.o.i. of 10. After 1 h incubation at 28°C on a shaking platform, the cells were pelleted at 100 *g*; the old medium was replaced by 50 ml of fresh medium, and the cells were resuspended and incubated further at 28°C on a shaking platform. At 2, 3, 4, and 5 d.p.i., aliquots containing 3×10^6 total cells were taken and centrifuged at 580 *g* and the supernatant removed. The cell pellets were resuspended in either 180 μl PBS or 180 μl PBS containing 30 μg/ml E-64 and then sonicated using a Branson Sonifier 250 at an output control setting of 5 for 5 s on ice. A portion of these lysates (19 μl each) were saved at −80°C for later Western blot analysis; E-64 was added to a final concentration of 30 μg/ml prior to freezing. Another portion (83 μl) was used to measure proteolytic activity immediately. Each 83 μl aliquot was added to 583 μl of 1.1% (w/v) azocasein in 125 mM sodium phosphate, pH 7.0, with or without E-64 (final concentration of 30 μg/ml). Reactions were incubated at 37°C for 90 min and then halted by addition of 27 μl of 100% trichloroacetic acid. Samples were centrifuged twice at 15,000 *g* for 5 min to remove undigested substrate, and each time the supernatant was transferred to new tubes. Finally, 333 μl 9 M urea was added to each sample to enhance the solubility of proteolytic fragments prior to measuring absorbance at 410 nm.

Analysis of live and dead cell populations

Sf9 cells were infected with wild-type E2 virus as described above. When approximately 90% of the cells had died from the infection 4–5 d.p.i. (as determined by trypan blue exclusion), the cells were collected by centrifugation (10 min at 1000 *g*) and resuspended in fresh medium at a concentration of 1×10^7 cells/ml. After filtering through 80-μm nylon mesh to remove clumps, cells were stained with propidium iodide (final concentration, 5 μg/ml). Fluorescence-activated cell sorting was then used to isolate fluorescing (dead) cells (λ_{ex} = 535 nm, λ_{em} = 617 nm) and nonfluorescing (live) cells. Trypan blue staining confirmed that cells had been sorted appropriately. The cells in the two fractions were then collected by centrifugation, resuspended in lysis buffer, and analyzed by Western blot as described above.

Comparative mortality rates of infected cells

Suspension cultures (15 ml) of log phase Sf9 and TN368 cells were infected with either wild-type L1 or

C^-DZ1 at an m.o.i. of 10. At 2, 3, 4, and 5 d.p.i., three separate 46-μl aliquots were removed, mixed with an equal volume of trypan blue, and counted using a hemocytometer. Dead cells were identified by their inability to exclude the dye trypan blue [final concentration, 0.125%(w/v)]. The experiments were repeated twice.

Lysosomal staining

Glass coverslips were prepared by soaking overnight in 1% HCl/95% EtOH, followed by thorough rinsing in distilled water and baking for 1 h at 100°C. Sf9 cells were allowed to settle on the coverslips in cell culture dishes for 1 h and then were infected with wild-type budded virus as described above. At 92 h.p.i., cells were stained with acridine orange (5 μg/ml in Grace's medium) for 15 min. The coverslips were then mounted in Grace's medium on a glass slide and viewed using a Zeiss Axiophot fluorescence microscope.

Detection of V-CATH in infected cell medium

Cells were infected as described for the time course above, except that cells were placed in Excell 401 serum-free medium after removal of the inoculum to avoid problems associated with the overloading of serum proteins during electrophoresis. At the prescribed times postinfection, the cells were harvested as described above, and the infected cell medium was set aside. E-64 (final concentration, 30 μg/ml) was added to prevent activation of or proteolysis by V-CATH contained in the medium. After centrifuging the medium for 30 min in a Beckman airfuge at 22 psi to remove small particulates (such as budded virus), 2 vol of cold acetone was added to precipitate the proteins in the medium. After incubating at −20°C for 30 min, the proteins were pelleted by centrifugation for 30 min at 15,000 *g*, resuspended in lysis buffer, and analyzed by Western blot.

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